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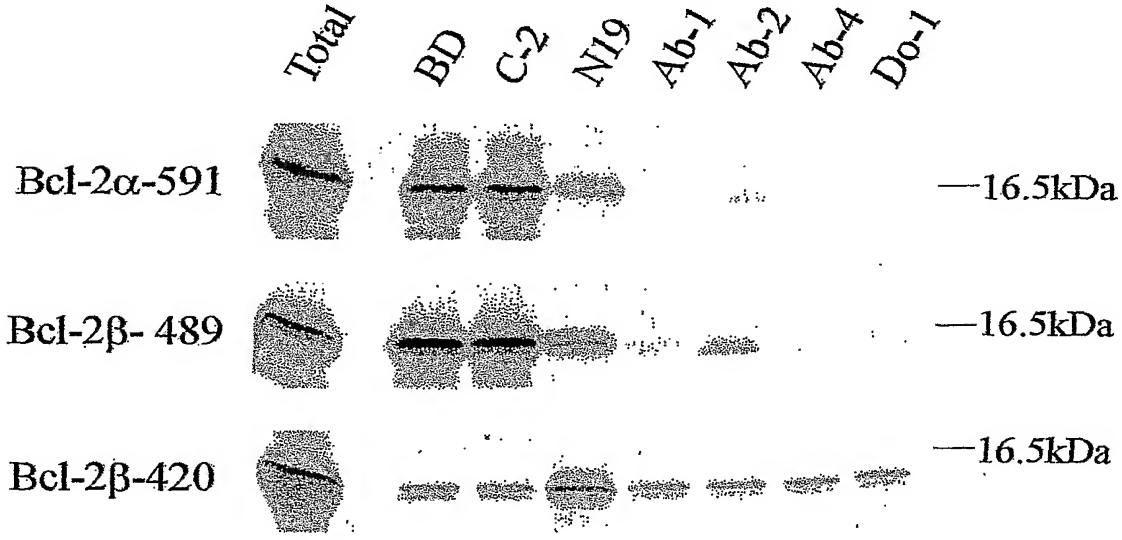
(54) Title: BCL-2 SPLICING VARIANTS



(57) Abstract: The present invention provides a method of regulating apoptosis in a cell and comprises targeting an abnormally or alternatively spliced mRNA, an abnormally or alternatively structured mRNA, or a product of either. The invention also provides a nucleotide construct with a nucleotide sequence that is homologous to mRNA transcribed from an abnormally spliced gene and a pharmaceutical composition comprising the nucleotide construct in association with a pharmaceutically acceptable carrier.

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BCL-2 SPLICING VARIANTS

Field of the invention

This invention relates to transcripts of genes that encode regulators of mammalian cell viability and to the manipulation of cell viability through the targeting of variants of such transcripts.

Background to the invention

Mammalian cell viability is determined by a continual balance between pro- and anti-death signals. The best understood process is that of apoptopic cell death.

Bcl-2 is an inhibitor of apoptosis. The functions of the Bcl-2 protein include protection against mitochondrial changes associated with apoptosis. This is achieved by inhibiting pro-apoptotic proteins and by preventing mitochondrial permeability transition. Apoptosis can be triggered by release of cytochrome c and other pro-apoptotic components from the mitochondria: Bcl-2 is believed to inhibit such events. Consistent with these functions the Bcl-2 protein is predominantly localised to the mitochondria. Bcl-2 may also have additional anti-apoptotic functions yet to be described. It may also block mitochondrial-independent pathways involved in apoptosis.

The human Bcl-2 gene encodes mRNA transcripts of (i) 720 nucleotides in length for Bcl-2α and (ii) of 618 nucleotides in length for Bcl-2β (see Figure 1). Bcl-2α and Bcl-2β represent normal, alternatively spliced variants of the same Bcl-2 gene. Abnormal and/or constitutive expression of functional Bcl-2 can protect mammalian

cells from undergoing apoptosis. Such an effect favours continued cell survival and proliferation, and can initiate and/or maintain abnormal and/or cancerous growth.

In colorectal cancer cells evidence for a novel Bcl-2 – p53 axis has been reported for a number of established human colorectal carcinoma cells lines, including the LoVo and SW48 cell lines. Co-pending patent application GB0306148.8 relates to the silencing of Bcl-2 by RNA interference. p53-dependent apoptosis is induced indicating that Bcl-2 constitutively suppresses a pro-apoptotic function of p53 in colorectal cancer cells. Importantly, this pro-apoptotic function of p53 does not require activation of the p53 protein by genotoxic stress or by other means. Constitutive Bcl-2 suppression of p53-dependent apoptosis is likely to contribute to the survival of human colorectal cancer cells.

There is a need to identify cell growth control targets for treating malignancies in humans and other mammalian species.

Statements of the invention

According to the present invention there is provided a method of regulating apoptosis in a cell, said method comprising targeting an abnormally or alternatively spliced mRNA, an abnormally or alternatively structured mRNA, or a product of either.

The term 'regulate' is used to refer to the situation where the threshold of apoptosis in a cell is controlled or adjusted to a particular specification or requirement and may refer to either 'up regulation', wherein the threshold of apoptosis is increased as

compared to that which is observed in said cell, in absence of performance of the method, or down regulation, where the threshold of apoptosis is decreased as compared to that which is observed in said cell, in absence of performance of the method.

Within genes, DNA serves as a template for the production of messenger RNA, which in turn is a template for the production of proteins. Messenger RNA molecules typically contain protein-coding regions called "exons" as well as non protein-coding regions called 'introns'.

It is known that mammalian RNA transcripts are modified in the nucleus by additions to the 5' and 3' ends of the molecule and by internal splicing to remove the introns. The splicing event requires breakage of the exon-intron junctions and joining of the ends of the exons. By comparing the nucleotide sequence of mRNA with that of the structural gene, the junctions between exons and introns can be assigned. The junctions have well conserved, though rather short consensus sequences. The really high conservation is found only immediately within the intron at the presumed junctions. An intron starts with the dinucleotide GT and ends with the dinucleotide AG. Accordingly, the junctions are often described as conforming to the GT-AG rule. The GT-AG rule describes the splicing sites of nuclear genes of many (perhaps all) eukaryotes.

However, the above is a very simplistic view of gene splicing. With the advent of information generated by various genome sequencing programmes it is evident that alternative pre-mRNA splicing is frequently used to expand the coding capacity of genomes. Splicing motifs are being continually discovered and tissue specific splicing patterns are emerging. Exonic splicing silencers (ESS) are exonic cis-regulatory elements that inhibit splicing, often leading to exon skipping. Thus the permutations of genetic information expressed via a single gene can be amplified and regulated.

The Bc1-2 gene encodes 3 exons. The interspersed introns must be precisely snipped away from Bcl-2 messenger RNA and the remaining exons must be accurately spliced together, with no 'exon skipping', if a normal Bcl-2 protein is to be produced. The splicing machinery in the cell nucleus cuts and pastes (due to a "lariat" intermediate) to generate a single, properly spliced Bcl-2 messenger RNA molecule. Normally two alternative splice variants are detected, Bcl-2α and Bcl-2β, to give protein products of 239 and 205 amino acids respectively.

Exon skipping due to mutations in Bcl-2 and many other genes is frequently, if not always, caused by the disruption of "exonic splicing enhancers," or ESEs. ESEs are sequences within exons that stimulate messenger RNA splicing. Diverse mutations in genes lead to RNA splicing defects and in turn, to various diseases.

The term 'abnormally or alternatively spliced' is used interchangeably to refer to the situation where mRNA is spliced using a splice sequence not normally used in processing the normal transcript(s) and the resulting mRNA sequence is different to that of the full-length normal sequence transcripts. Internal structures within the mRNA transcript, for example stem loops and pseudo knots, can also affect the information flow from transcript to translated protein product.

Preferably the method involves targeting the junctions of mRNA molecules that are abnormally or alternatively spliced or abnormally or alternatively structured.

The term 'junction' is used to refer to the particular nucleotide sequence that is created by the attachment or joining together of the alternatively spliced mRNA. The term 'junction' also encompasses the apparent junction created by the presence of secondary RNA structures within the mRNA: such structures can cause looping out from a lateral stem on the mRNA such that the apparent linear sequence resembles that of a spliced junction upon amplification by RT-PCR. For Bcl-2 mRNA from colorectal carcinoma cells evidence for such structures has been discovered using Abgene reverse blender RT-PCR amplification at 47°C; and the Two-step Sigma RT-PCR kit at 47°C. Both these procedures give shorter spliced Bcl-2 cDNAs and, importantly, the apparent splicing conserves the triplet reading frame for Bcl-2 mRNA (Figure 1). However, when RT-PCR is carried out using Quiagen sense-script reverse transcriptase at 50°C, the cDNA product is for full length Bcl-2 mRNA. These results indicate that Bcl-2 mRNA from colorectal cancer cells contains highly ordered loop structures.

Alternatively the method involves targeting a protein product following translation of an abnormally/alternatively spliced mRNA or abnormally/alternatively structured mRNA.

Preferably the method comprises selective silencing of abnormal splice variants of the Bcl-2 gene.

The term 'selectively silencing' is used to indicate that the silencing is specific for the target gene and that there is no interference with normal, endogenous gene expression which might be detrimental to normal non-cancerous cells.

Preferably the method involves the targeting of any of the abnormal splice variants Bcl-2α-591, Bcl-2α-588, Bcl-2α-480, Bcl-2α-633, Bcl-2β-489, Bcl-2β-474, Bcl-2β-420 and/or Bcl-2β-315. More preferably the method involves targeting the mRNA sequence flanking the splice junction between nucleotides 111 and 241 of Bcl-2α-591.

In one embodiment the method of the invention involves targeting an abnormally/alternatively spliced or abnormally/alternatively structured mRNA or a product of either, by introducing into a cell containing a gene which is abnormally spliced and which is to be targeted, an RNA construct having a nucleotide sequence which is homologous to mRNA within said cell wherein said mRNA includes genetic information of the gene element that is abnormally spliced.

Is is known that the introduction of dsRNA into cells initiates RNA interference (RNAi). RNAi induces sequence-specific degradation of homologous mRNA. In mammalian cells RNAi can be achieved using small interfering dsRNAs (siRNAs), preferably up to 28 nucleotides long and more preferably 21-22 nucleotides long.

The term 'homologous' is used to indicate at least 50%, preferably 85%, more preferably 90%, more preferably 95% and most preferably 100% homology to the reference nucleic acid sequence.

The present invention relates to the discovery of abnormal splice variants of Bcl-2 mRNA in human colorectal carcinoma cells. Sequence alignments are given in Figure 1. The novel splice junctions conserve the normal triplet framing of the spliced mRNA products and the functional BH1, BH2, BH3 and BH4 domains of the Bcl-2 protein are also conserved (where BH stands for 'Bcl-2 homology domain').

Abnormal alternatively spliced variants of Bcl-2 may function constitutively to suppress apoptosis in human and other mammalian cells, enabling abnormal cell survival and abnormal cell proliferation. The expression of abnormally spliced variants of Bcl-2 may thus represent a key oncogenic event in the development of cancer. The abnormal splice junctions of the Bcl-2 mRNA molecules represent selective targets for intervention via RNA interference or other means. The mRNA sequence at these abnormal splice junctions is not present in the normally spliced full length Bcl-2 mRNAs.

These abnormal Bcl-2 mRNA transcripts are shorter than the full length 'wild type' Bcl-2 mRNA. In contrast analysis of the genomic Bcl-2 by PCR amplification gives the predicted length for wild type Bcl-2 DNA (Figure 2). This indicates that the shorter abnormal Bcl-2 mRNA transcripts are indeed generated by alternative splicing of RNA, rather than genomic events with loss of DNA coding sequence from the human Bcl-2 gene.

The abnormal alternative spliced variants of Bcl-2 mRNA expressed in human colorectal cancer cells remain in-frame for the triplet genetic code and retain all known functional domains of the Bcl-2α and Bcl-2β proteins (see Figure 1) and are functional in the suppression of apoptosis. Functionality is also evident in colorectal carcinoma cell lines in which Bcl-2 expression may comprise solely of the abnormal alternative mRNA form(s). In such cells the selective silencing of Bcl-2 expression by RNA interference induces apoptosis (Jiang and Milner, 2003).

In one embodiment of the invention, selective silencing of alternatively spliced Bcl-2 expression is achieved by RNA interference. Alternatively silencing may be achieved by any other 'silencing means' such as small molecules, peptides and/or related molecules that inhibit Bcl-2 either directly or indirectly, and also Bcl-2 derived products including abnormal Bcl-2 splice variants. Anti-sense RNA, shRNA, miRNA and any other RNA and/or DNA based strategies may also be used. Tumour cells other than colorectal cancer cells may similarly be treated, such as ovarian cancer cells.

In one embodiment the present invention provides a nucleotide construct with a nucleotide sequence that is homologous to mRNA transcribed from an abnormally or alternatively spliced gene.

Preferably the nucleotide construct comprises dsRNA. Preferably the construct is 30 or less nucleotides long. More preferably the RNA construct is 20 to 30 nucleotides long. Most preferably the RNA construct is 21 to 22 nucleotides long.

In one embodiment the invention provides a nucleotide construct such as anti-sense RNA, shRNA or miRNA as means for silencing the expression of an abnormally or alternatively spliced gene for use as a medicament.

In an alternate embodiment the invention provides an agent selected from the group consisting of: small molecule or protein, polypeptide; peptide; aptamer; chemical; antibody; nucleic acid; polypeptide or nucleotide probe which agent interacts with or binds with a protein expressed by an abnormally spliced mRNA for use as a medicament. Preferably the agent comprises a sequence or molecular structure that is complimentary to or of sufficient homology to give specific binding to the target.

In an alternative embodiment the invention provides a nucleotide construct such as anti-sense RNA, shRNA or miRNA for the manufacture of a medicament for the treatment of cancerous cell growth.

In an alternate embodiment the invention provides an agent selected from the group consisting of: small molecule or protein, polypeptide; peptide; aptamer; chemical; antibody; nucleic acid; polypeptide or nucleotide probe which agent interacts with or binds with a protein expressed by an abnormally spliced mRNA for the manufacture of a medicament for the treatment of cancerous cell growth.

The invention also provides a pharmaceutical composition comprising a nucleotide construct such as anti-sense RNA, shRNA or miRNA and a pharmaceutically acceptable diluent or carrier.

In an alternate embodiment the invention provides an agent selected from the group consisting of: small molecule or protein, polypeptide; peptide; aptamer; chemical; antibody; nucleic acid; polypeptide or nucleotide probe which agent interacts with or binds with a protein expressed by an abnormally spliced mRNA and a pharmaceutically acceptable diluent or carrier.

According to a further aspect of the invention there is provided a DNA or RNA expression vector as a delivery means for, for example, an antisense or an RNAi molecule that is used in the targeting of an abnormally spliced mRNA or a product thereof.

In one embodiment of the invention a viral vector is used as delivery means.

Preferably the vector includes an expression cassette comprising the nucleotide sequence selected from the group consisting of;

a) the nucleic acid sequence of the abnormally spliced gene element as shown in Fig 1;

b) a nucleic acid molecule which hybridizes to the nucleic acid sequence of (a) ;

c) a nucleic acid molecule which has a nucleic acid sequence which is degenerate because of the genetic code to the sequences in a) and b) and any sequence which is complimentary to any of the above sequences;

wherein the expression cassette is transcriptionally linked to a promoter sequence.

Preferably the vector including the expression cassette is adapted for eukaryotic gene expression. Typically said adaptation includes, by example and not by way of limitation, the provision of transcription control sequences (promoter sequences) which mediate cell/tissue specific expression. These promoter sequences may be cell/tissue specific, inducible or constitutive.

Promoter elements typically also include so called TATA box and RNA polymerase initiation selection sequences which function to select a site of transcription initiation. These sequences also bind polypeptides that function, *inter alia*, to facilitate transcription initiation selection by RNA polymerase.

Adaptations also include the provision of selectable markers and autonomous replication sequences which both facilitate the maintenance of said vector in either the eukaryotic cell or prokaryotic host. Vectors that are maintained autonomously are referred to as episomal vectors. Further adaptations which facilitate the expression of vector encoded genes include the provision of transcription termination sequences.

These adaptations are well known in the art. There is a significant amount of published literature with respect to expression vector construction and recombinant DNA techniques in general. Please see, Sambrook et al (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory, Cold Spring Harbour, NY and references therein; Marston, F (1987) DNA Cloning Techniques: A Practical Approach Vol III IRL Press, Oxford UK; DNA Cloning: F M Ausubel et al, Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994).

Detailed Description of the Invention

The present invention will now be described by way of example only and with reference to the following diagrams;

Figure 1

Sequence alignments of human Bcl-2 splice variants in colorectal cell lines (including LoVo; SW48 and HCT116). Boxed areas indicate functional domains of Bcl-2. Note that Bcl-2α-591; -α588; -α480; -α633 and Bcl-2β-489; -β474; -β420 and –β315 retain all functional domain sequences. Dashes indicate missing sequences from abnormally spliced Bcl-2 variants.

Figure 2

Sizing of Bcl-2 genomic DNA following PCR amplification from individual human colorectal cell lines as indicated, using primers designed to span all abnormal splice sites identified to date. The predicted size for the intact genomic Bcl-2 DNA PCR-generated sequence, using the chosen primers, is 570 base pairs. This is the size observed in all colorectal cell lines tested to date, as indicated on the gels. [Note that genomic Bcl-2 is normally only spliced to generate the Bcl-2α and Bcl-2β variants].

Figure 3

Expression of abnormal alternatively spliced variants of human Bcl-2 in vitro and immunoprecipitation with anti-Bcl-2 antibodies. Bcl-2 mRNA from human colorectal cancer cells was reverse transcribed to produce a cDNA template from which cRNA was transcribed and translated. Translation was performed in the presence of 35S-methionine and radiolabelled protein was visualised by autoradiography following immunoprecipitation and resolution by SDS-PAGE. Three abnormal splice variants are shown (Bcl-2α-591; Bcl-2β-489; and Bcl-2β-420 as indicated).

Figure 4

Table summarising the truncated RNA products derived from the Bcl-2 gene and detected by PCR in samples of different colorectal cell lines. HCT = HCT116 with six individual clones which fall into three isogenic pairs with knock-out for p53; p21 and Bax genes, as indicated.

Figure 5

Radiolabelled Bcl-2 proteins following in vitro transcription and translation using rabbit reticulocyte lysate. Proteins were resolved by SDS-PAGE and visualised by autoradiography. Upper band = full length Bcl-2α; lower bands = protein products generated via alternatively spliced/alternatively structured Bcl-2 mRNA.

Materials and Methods

Bcl2 detection and cloning by RT-PCR

The primers used for Bcl2 amplification in colon cancer cell lines were as follows:

	5'→ 3'
Bcl-2up	ccatcgatggcgcacgctgggagaac
Bcl-2dn(α)	ccggaattcacttgtggcccagatagg
Bcl-2dn(β)	ccggaattcagcccagactcacatcacca
Bcl-2up2	ccgggagatagtgatgaagtaca
Bcl-2dn2	cctggatccaggtgtgcaggt
Bcl-2dn3	tgccggttcaggtactcagtc

The RT-PCR is performed using 100ng total RNA, bcl2up and bcl2dn with one-step RT-PCR kit from ABgene (cat. AB-0844 or AB-0844/b) in a thermal cycle as follows: 47°C 30min, 94°C 2min, then 35 cycles of 94°C, 45sec, 58°C, 45sec, 72°C, 1min, followed by 72°C for 5min. There are only shortened Bcl-2 products amplified using above method. The full-length Bcl-2 product can only be amplified using Qiagen one-step RT-PCR kit in a thermal cycle as below: 50°C 30min, 94°C 15min,

then 35 cycles of 94°C, 45sec, 58°C, 45sec, 72°C, 1min, followed by 72°C for 10min.

The PCR products were purified and digested with E*coR*I and C*la*I, and cloned into pBSK$^+$, then transcribed using T7 polymerase and translated using Promega RRL in vitro. The translated protein were immuno-precipitated with various Bcl-2 antibodies: BD (BD biosciences), C-2, N-19 (Satan Cruz), Ab-1, Ab-2, and Ab-4 (Oncogene).

Bcl-2 antibodies employed in this study – positions of their epitopes:

Bcl-2 (BD) Against 49-179aa. From current study, it can be refined to 81-88aa.

Bcl-2 (C-2) Against a recombinant protein corresponding to amino acids 1-205. From the current study, it can be refined to 81-88aa.

Bcl-2 (N-19) Against a peptide mapping at the amino terminus of Bcl-2. From the current study, it can map at 1-23aa.

Bcl-2 (Ab-1) 41-54aa.

Bcl-2 (Ab-2) 20-34aa.

Bcl-2 (Ab-4) 61-76aa.

Cloning and expression of abnormal alternative splice variants of Bcl-2 in vitro.

Abnormal alternative splice/structural variants of Bcl-2 mRNAs have been cloned from colorectal cancer cells and expressed in vitro. The results demonstrate that the abnormal alternative splice/structural variants of Bcl-2 are expressed as protein (Figure 3).

Lack of specific Bcl-2 epitopes was observed for the protein products encoded by the abnormal alternatively spliced Bcl-2 variants. Abnormal splicing in some way interferes with epitope availability for antibody recognition. It is proposed that epitope loss may prove to be a useful indicator of alternatively spliced Bcl-2 expression. For example, the variant Bcl-2α-591 appears to contain a novel splice junction between nucleotides 111 and 241 (Figure 1): the protein expressed endogenously from this splice variant in human cells reacts poorly with the N19 anti-Bcl-2 antibody in immunoblots (Jiang and Milner, 2003), and in immunoprecipitation reactions following its expression in vitro (Figure 3). Loss of antibody reactivity may also be evident in tissue sections stained by immunocytochemistry. Epitope loss or modification may prove to be of clinical and diagnostic importance for identifying the expression of abnormal alternative spliced variants of Bcl-2 in human tissues. The same principles apply to tissues of other mammalian species.

Alternative abnormal spliced variants of Bcl-2 may represent a tumour-related abnormality. This abnormality may not be restricted to cancers arising from

colorectal epithelial cells. Other tumour types may also be affected, including other epithelial tumours and/or tumours/malignancies arising from other cell types. Any tumour-related abnormality represents a promising target for selective therapy designed to selectively target malignancies in humans and in other mammalian species. Such therapies may, in principle, be designed to suppress gene expression using, for example, RNA interference. An alternative approach would be to target abnormal mRNA structures using selective binding molecules. An alternative approach would be to target functional protein-protein interactions by, for example, small molecules designed to disrupt essential molecular interfaces between the Bcl-2 protein and its functional protein partners. Any differences in protein structure created as a result of abnormal alternative splicing of Bcl-2 mRNA represent potential tumour-specific targets for novel anti-cancer molecules and/or other reagents.

References:

1. **Jiang M & Milner J.** Bcl-2 constitutively suppresses p53-dependent apoptosis in colorectal cancer cells. **Genes & Development,** 17; 832-837 (2003).

Claims

1. A method of regulating apoptosis in a cell, said method comprising targeting an abnormally or alternatively spliced mRNA, an abnormally or alternatively structured mRNA, or a product of either.

2. A method according to claim 1 further comprising targeting the junctions of the mRNA molecule that is abnormally spliced or abnormally structured.

3. A method according to claim 1 further comprising targeting a protein product following translation of the abnormally spliced or abnormally structured mRNA.

4. A method according to any of claims 1 to 3 further comprising the selective silencing of abnormal splice variants of the Bcl-2 gene.

5. A method according to claim 4 further comprising the targeting of any of the abnormal splice variants selected from the group consisting of: Bcl-2α-591, Bcl-2α-588, Bcl-2α-480, Bcl-2α-633, Bcl-2β-489, Bcl-2β-474, Bcl-2β-420 and/or Bcl-2β-315.

6. A method according to claim 5 further comprising targeting of the mRNA sequence flanking the splice junction between nucleotides 111 and 241 of Bcl-2α-591.

7. A method according to any of the preceding claims further comprising targeting an abnormally spliced mRNA or a product thereof, by introducing into a cell containing a gene which is abnormally spliced and which is to be targeted, an RNA construct having a nucleotide sequence which is homologous to mRNA within said cell wherein said mRNA includes genetic information of the gene element that is abnormally spliced.

8. A method according to claim 7 wherein the RNA construct is a small interfering dsRNA (siRNA).

9. A method according to claim 8 wherein the siRNA is up to 28 nucleotides long.

10. A method according to any of claims 1 to 6, further comprising targeting an abnormally spliced mRNA or a product thereof, by introducing into a cell containing a gene which is abnormally spliced and which is to be targeted, an agent selected from the group consisting of: small molecule or protein; polypeptide; peptide; aptamer; chemical; antibody; nucleic acid; polypeptide or nucleotide probe; anti-sense RNA; shRNA; miRNA; and Bcl-2 derived products including abnormal Bcl-2 splice variants which inhibit Bcl-2 either directly or indirectly, which agent interacts with or binds with the abnormally spliced mRNA or protein expressed by the abnormally spliced mRNA.

11. A nucleotide construct with a nucleotide sequence which is homologous to mRNA transcribed from an abnormally spliced gene.

12. A nucleotide construct according to claim 11 wherein said construct comprises dsRNA.

13. A nucleotide construct according to claim 12 wherein the construct is 20 to 28 nucleotides long.

14. A nucleotide construct according to claim 13 wherein the RNA construct is 21 to 22 nucleotides long.

15. A nucleotide construct such as siRNA, anti-sense RNA, shRNA or miRNA as means for silencing the expression of an abnormally spliced gene for use as a medicament.

16. An agent selected from the group consisting of: small molecule or protein; polypeptide; peptide; aptamer; chemical; antibody; nucleic acid; polypeptide or nucleotide probe, which agent interacts with or binds with a protein expressed by an abnormally spliced mRNA for use as a medicament.

17. A nucleotide construct such as siRNA, anti-sense RNA, shRNA or miRNA for the manufacture of a medicament for the treatment of cancerous cell growth..

18. An agent selected from the group consisting of: small molecule or protein; polypeptide; peptide; aptamer; chemical; antibody; nucleic acid; polypeptide or nucleotide probe which agent interacts with or binds with a protein expressed by an abnormally spliced mRNA for the manufacture of a medicament for the treatment of cancerous cell growth.

19. A pharmaceutical composition comprising a nucleotide construct such as siRNA, anti-sense RNA, shRNA or miRNA and a pharmaceutically acceptable diluent or carrier.

20. A pharmaceutical composition comprising an agent selected from the group consisting of: small molecule or protein; polypeptide; peptide; aptamer; chemical; antibody; nucleic acid; polypeptide or nucleotide probe which agent interacts with or binds with a protein expressed by an abnormally spliced mRNA and a pharmaceutically acceptable diluent or carrier.

21. Use of a DNA or RNA expression vector as a delivery means for a molecule which is used in the targeting of an abnormally spliced mRNA or a product thereof.

22. A DNA or RNA expression vector comprising an expression cassette including the nucleotide sequence selected from the group consisting of;

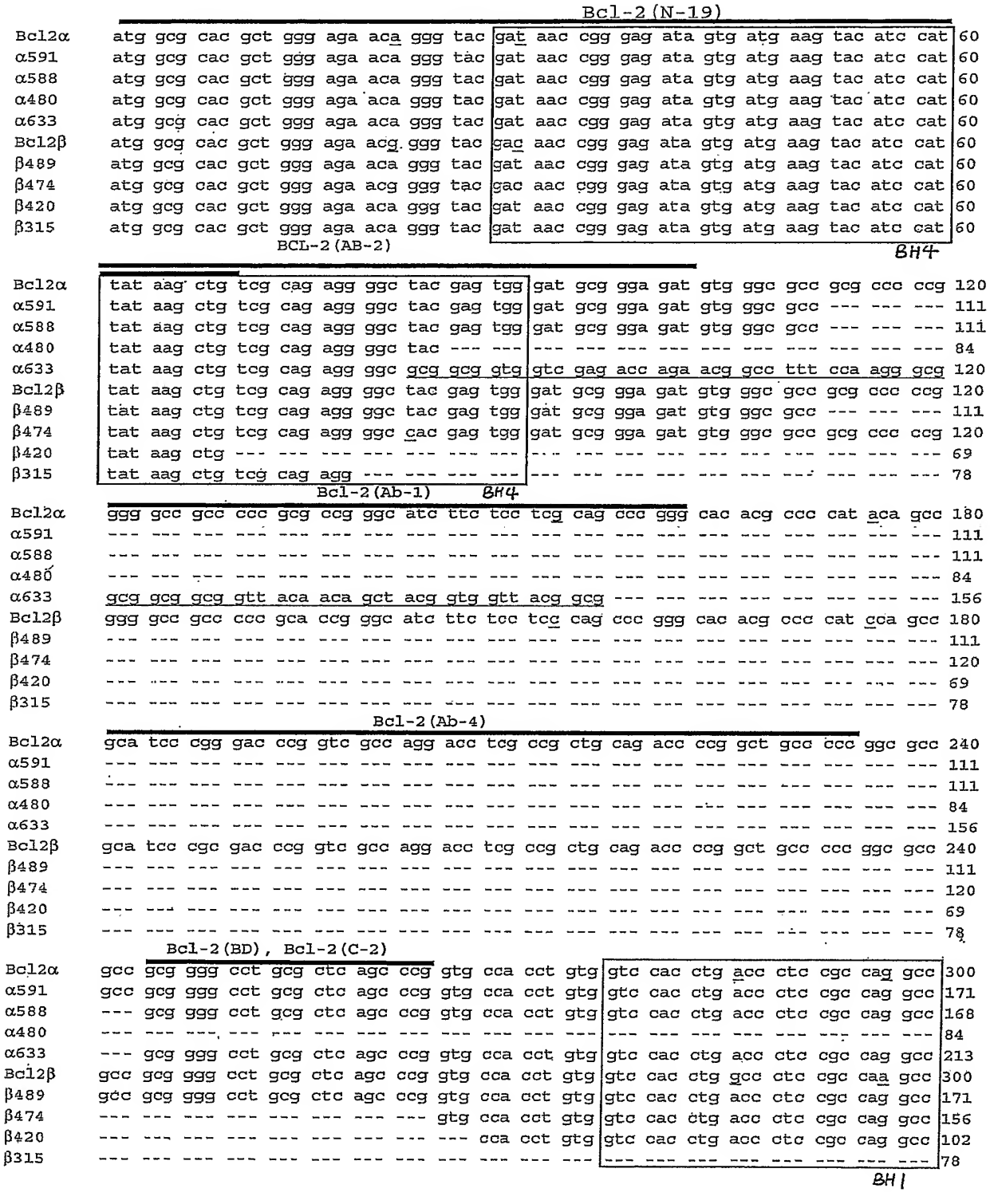
a) the nucleic acid sequence of the abnormally spliced gene element as shown in Fig 1;

b) a nucleic acid molecule which hybridizes to the nucleic acid sequence of (a) ;

c) a nucleic acid molecule which has a nucleic acid sequence which is degenerate because of the genetic code to the sequences in a) and b) and any sequence which is complimentary to any of the above sequences;

wherein the expression cassette is transcriptionally linked to a promoter sequence.

Figure 1


Bcl-2(N-19)
Bcl2α atg gcg cac gct ggg aga aca ggg tac gat aac cgg gag ata gtg atg aag tac atc cat 60
α591 atg gcg cac gct ggg aga aca ggg tac gat aac cgg gag ata gtg atg aag tac atc cat 60
α588 atg gcg cac gct ggg aga aca ggg tac gat aac cgg gag ata gtg atg aag tac atc cat 60
α480 atg gcg cac gct ggg aga aca ggg tac gat aac cgg gag ata gtg atg aag tac atc cat 60
α633 atg gcg cac gct ggg aga aca ggg tac gat aac cgg gag ata gtg atg aag tac atc cat 60
Bcl2β atg gcg cac gct ggg aga acg ggg tac gac aac cgg gag ata gtg atg aag tac atc cat 60
β489 atg gcg cac gct ggg aga aca ggg tac gat aac cgg gag ata gtg atg aag tac atc cat 60
β474 atg gcg cac gct ggg aga acg ggg tac gac aac cgg gag ata gtg atg aag tac atc cat 60
β420 atg gcg cac gct ggg aga aca ggg tac gat aac cgg gag ata gtg atg aag tac atc cat 60
β315 atg gcg cac gct ggg aga aca ggg tac gat aac cgg gag ata gtg atg aag tac atc cat 60
BCL-2(AB-2)
BH4
Bcl2α tat aag ctg tcg cag agg ggc tac gag tgg gat gcg gga gat gtg ggc gcc gcg ccc ccg 120
α591 tat aag ctg tcg cag agg ggc tac gag tgg gat gcg gga gat gtg ggc gcc --- --- --- 111
α588 tat aag ctg tcg cag agg ggc tac gag tgg gat gcg gga gat gtg ggc gcc --- --- --- 111
α480 tat aag ctg tcg cag agg ggc tac --- --- --- --- --- --- --- --- --- --- --- --- 84
α633 tat aag ctg tcg cag agg ggc gcg gcg gtg gtc gag acc aga acg gcc ttt cca agg gcg 120
Bcl2β tat aag ctg tcg cag agg ggc tac gag tgg gat gcg gga gat gtg ggc gcc gcg ccc ccg 120
β489 tat aag ctg tcg cag agg ggc tac gag tgg gat gcg gga gat gtg ggc gcc --- --- --- 111
β474 tat aag ctg tcg cag agg ggc cac gag tgg gat gcg gga gat gtg ggc gcc gcg ccc ccg 120
β420 tat aag ctg --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- 69
β315 tat aag ctg tcg cag agg --- --- --- --- --- --- --- --- --- --- --- --- --- --- 78
Bcl-2(Ab-1)
BH4
Bcl2α ggg gcc gcc ccc gcg ccg ggc atc ttc tcc tcg cag ccc ggg cac acg ccc cat aca gcc 180
α591 --- 111
α588 --- 111
α480 --- 84
α633 gcg gcg gcg gtt aca aca gct acg gtg gtt acg gcg --- --- --- --- --- --- --- --- 156
Bcl2β ggg gcc gcc ccc gca ccg ggc atc ttc tcc tcc cag ccc ggg cac acg ccc cat gca gcc 180
β489 --- 111
β474 --- 120
β420 --- 69
β315 --- 78
Bcl-2(Ab-4)
Bcl2α gca tcc cgg gac ccg gtc gcc agg acc tcg ccg ctg cag acc ccg gct gcc ccc ggc gcc 240
α591 --- 111
α588 --- 111
α480 --- 84
α633 --- 156
Bcl2β gca tcc cgc gac ccg gtc gcc agg acc tcg ccg ctg cag acc ccg gct gcc ccc ggc gcc 240
β489 --- 111
β474 --- 120
β420 --- 69
β315 --- 78
Bcl-2(BD), Bcl-2(C-2)
Bcl2α gcc gcg ggg cct gcg ctc agc ccg gtg cca cct gtg gtc cac ctg acc ctc cgc cag gcc 300
α591 gcc gcg ggg cct gcg ctc agc ccg gtg cca cct gtg gtc cac ctg acc ctc cgc cag gcc 171
α588 --- gcg ggg cct gcg ctc agc ccg gtg cca cct gtg gtc cac ctg acc ctc cgc cag gcc 168
α480 --- 84
α633 --- gcg ggg cct gcg ctc agc ccg gtg cca cct gtg gtc cac ctg acc ctc cgc cag gcc 213
Bcl2β gcc gcg ggg cct gcg ctc agc ccg gtg cca cct gtg gtc cac ctg gcc ctc cgc caa gcc 300
β489 gcc gcg ggg cct gcg ctc agc ccg gtg cca cct gtg gtc cac ctg acc ctc cgc cag gcc 171
β474 --- --- --- --- --- --- --- --- gtg cca cct gtg gtc cac ctg acc ctc cgc cag gcc 156
β420 --- --- --- --- --- --- --- --- --- cca cct gtg gtc cac ctg acc ctc cgc cag gcc 102
β315 --- 78
BH1

```
Bcl2α   ggc gac gac ttc tcc cgc cgc tac cgc cgc gac ttc gcc gag atg tcc agg cag ctg cac 360
α591    ggc gac gac ttc tcc cgc cgc tac cgc cgc gac ttc gcc gag atg tcc agc cag ctg cac 231
α588    ggc gac gac ttc tcc cgc cgc tac cgc cgc gac ttc gcc gag atg tcc agc cag ctg cac 228
α480    --- --- --- --- --- --- --- --- cgc cgc gac ttc gcc gag atg tcc agc cag ctg cac 120
α633    ggc gac gac ttc tcc cgc cgc tac cgc cgc gac ttc gcc gag atg tcc agc cag ctg cac 273
Bcl2β   ggc gac gac ttc tcc cgc cgc tac cgc ggc gac ttc gcc gag atg tcc agc cag ctg cac 360
β489    ggc gac gac ttc tcc cgc cgc tac cgc cgc gac ttc gcc gag atg tcc agc cag ctg cac 231
β474    ggc gac gac ttc tcc cgc cgc tac cgc cgc gac ttc gcc gag atg tcc agc cag ctg cac 216
β420    ggc gac ggc ttc tcc cgc cgc tac cgc cgc gac ttc gcc gag atg tcc agc cag ctg cac 162
β315    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- 78
                                    BH1

Bcl2α   ctg acg ccc ttc acc gcg cgg gga cgc ttt gcc acg gtg gtg gag gag ctc ttc agg gac 420
α591    ctg acg ccc ttc acc gcg cgg gga cgc ttt gcc acg gtg gtg gag gag ctc ttc agg gac 291
α588    ctg acg ccc ttc acc gcg cgg gga cgc ttt gcc acg gtg gtg gag gag ctc ttc agg gac 288
α480    ctg acg ccc ttc acc gcg cgg gga cgc ttt gcc acg gtg gtg gag gag ctc ttc agg gac 180
α633    ctg acg ccc ttc acc gcg cgg gga cgc ttt gcc acg gtg gtg gag gag ctc ttc agg gac 333
Bcl2β   ctg acg ccc ttc acc gcg cgg gga cgc ttt gcc acg gtg gtg gag gag ctc ttc agg gac 420
β489    ctg acg ccc ttc acc gcg cgg gga cgc ttt gcc acg gtg gtg gag gag ctc ttc agg gac 291
β474    ctg acg ccc ctc acc gcg cgg gga cgc ttt gcc acg gtg gtg gag gag ctc ttc agg gac 276
β420    ctg acg ccc ttc acc gcg cgg gga cgc ttt gcc tcg gtg gtg gag gag ctc ttc agg gac 222
β315    --- --- --- --- --- --- --- gga cgc ttt gcc acg gtg gtg gag gag ctc ttc agg gac 117
                                                                    BH2

Bcl2α   ggg gtg aac tgg ggg agg att gtg gcc ttc ttt gag ttc ggt ggg gtc atg tgt gtg gag 480
α591    ggg gtg aac tgg ggg agg att gtg gcc ttc ttt gag ttc ggt ggg gtc atg tgt gtg gag 351
α588    ggg gtg aac tgg ggg agg att gtg gcc ttc ttt gag ttc ggt ggg gtc atg tgt gtg gag 348
α480    ggg gtg aac tgg ggg agg att gtg gcc ttc ttt gag ttc ggt ggg gtc atg tgt gtg gag 240
α633    ggg gtg aac tgg ggg agg att gtg gcc ttc ttt gag ttc ggt ggg gtc atg tgt gtg gag 393
Bcl2β   ggg gtg aac tgg ggg agg att gtg gcc ttc ttt gag ttc ggt ggg gtc atg tgt gtg gag 480
β489    ggg gtg aac tgg ggg agg att gtg gcc ttc ttt gag ttc ggt ggg gtc atg tgt gtg gag 351
β474    ggg gtg aac tgg ggg agg att gtg gcc ttc ttt gag ttc ggt ggg gtc atg tgt gtg gag 336
β420    ggg gtg aac tgg ggg agg att gtg gcc ttc ttt gag ttc ggt ggg gtc atg tgt gtg gag 282
β315    ggg gtg aac tgg ggg agg att gtg gcc ttc ttt gag ttc ggt ggg gtc atg tgt gtg gag 177
                                                            BH2

Bcl2α   agc gtc aac cgg gag atg tcg ccc ctg gtg gac aac atc gcc ctg tgg atg act gag tac 540
α591    agc gtc aac cgg gag atg tca ccc ctg gtg gac aac atc gcc ctg tgg atg act gag tac 411
α588    agc gtc aac cgg gag atg tcg ccc ctg gtg gac aac atc gcc ctg tgg atg act gag tac 408
α480    agc gtc aac cgg gag atg tcg ccc ctg gtg gac aac atc gcc ctg tgg atg act gag tac 300
α633    agc gtc aac cgg gag atg tcg ccc ctg gtg gac aac atc gcc ctg tgg atg act gag tac 453
Bcl2β   agc gtc aac cgg gag atg tcg ccc ctg gtg gac aac atc gcc ctg tgg atg act gag tac 540
β489    agc gtc aac cgg gag atg tcg ccc ctg gtg gac aac atc gcc ctg tgg atg act gag tac 411
β474    agc gcc aac cgg gag atg tcg ccc ctg gtg gac aac atc gcc ctg tgg atg act gag tac 396
β420    agc gtc aac cgg gag atg tcg ccc ctg gtg gac aac atc gcc ctg tgg atg act gag tac 342
β315    agc gtc aac cgg gag atg tcg ccc ctg gtg gac aac atc gcc ctg tgg atg act gag tac 237

Bcl2α   ctg aac cgg cac ctg cac acc tgg atc cag gat aac gga ggc tgg --- --- --- --- --- 585
α591    ctg aac cgg cac ctg cac acc tgg atc cag gat aac gga ggc tgg --- --- --- --- --- 456
α588    ctg aac cgg cac ctg cac acc tgg atc cag gat aac gga ggc tgg --- --- --- --- --- 453
α480    ctg aac cgg cac ctg cac acc tgg atc cag gat aac gga ggc tgg --- --- --- --- --- 345
α633    ctg aac cgg cac ctg cac acc tgg atc cag gat aac gga ggc tgg --- --- --- --- --- 498
Bcl2β   ctg aac cgg cac ctg cac acc tgg atc cag gat aac gga ggc tgg gta ggt gca tct ggt 600
β489    ctg aac cgg cac ctg cac acc tgg atc cag gat aac gga ggc tgg gta ggt gca tct ggt 471
β474    ctg aac cgg cac ctg cac acc tgg atc cag gat aac gga ggc tgg gta ggt gca tct ggt 456
β420    ctg aac cgg cac ctg cac acc tgg atc cag gat aac gga ggc tgg gta ggt gca tct ggt 402
β315    ctg aac cgg cac ctg cac acc tgg atc cag gat aac gga ggc tgg gta ggt gca tct ggt 297
                                                            BH3

Bcl2α   --- --- --- --- --- --- gat gcc ttt gtg gaa ctg tac ggc ccc agc atg cgg cct ctg 627
α591    --- --- --- --- --- --- gat gcc ttt gtg gaa ctg tac ggc ccc agc atg cgg cct ctg 498
α588    --- --- --- --- --- --- gat gcc ttt gtg gaa ctg tac ggc ccc agc atg cgg cct ctg 495
α480    --- --- --- --- --- --- gat gcc ttt gtg gaa ctg tac ggc ccc agc atg cgg cct ctg 387
α633    --- --- --- --- --- --- gat gcc ttt gtg gaa ctg tac ggc ccc agc atg cgg cct ctg 540
Bcl2β   gat gtg agt ctg ggc tga                                                         618
                                        BH3
β489    gat gtg agt ctg ggc tga                                                         489
β474    gat gtg agt ctg ggc tga                                                         474
β420    gat gtg agt ctg ggc tga                                                         420
β315    gat gtg agt ctg ggc tga                                                         315
```

```
Bcl2α    ttt gat ttc tcc tgg ctg tct ctg aag act ctg ctc agt ttg gcc ctg gtg gga gct tgc 687
α591     ttt gat ttc tcc tgg ctg tct ctg aag act ctg ctc agt ttg gcc ctg gtg gga gct tgc 558
α588     ttt gat ttc tcc tgg ctg tct ctg aag act ctg ctc agt ttg gcc ctg gtg gga gct tgc 555
α480     ttt gat ttc tcc tgg ctg tct ctg aag act ctg ctc agt ttg gcc ctg gtg gga gct tgc 447
α633     ttt gat ttc tcc tgg ctg tct ctg aag act ctg ctc agt ttg gcc ctg gtg gga gct tgc 600

Bcl2α    atc acc ctg ggt gcc tat ctg ggc cac aag tga                                  720
α591     atc acc ctg ggt gcc tat ctg ggc cac aag tga                                  591
α588     atc acc ctg ggt gcc tat ctg ggc cac aag tga                                  588
α480     atc acc ctg ggt gcc tat ctg ggc cac aag tga                                  480
α633     atc acc ctg ggt gcc tat ctg ggc cac aag tga                                  633
```

FIGURE 2

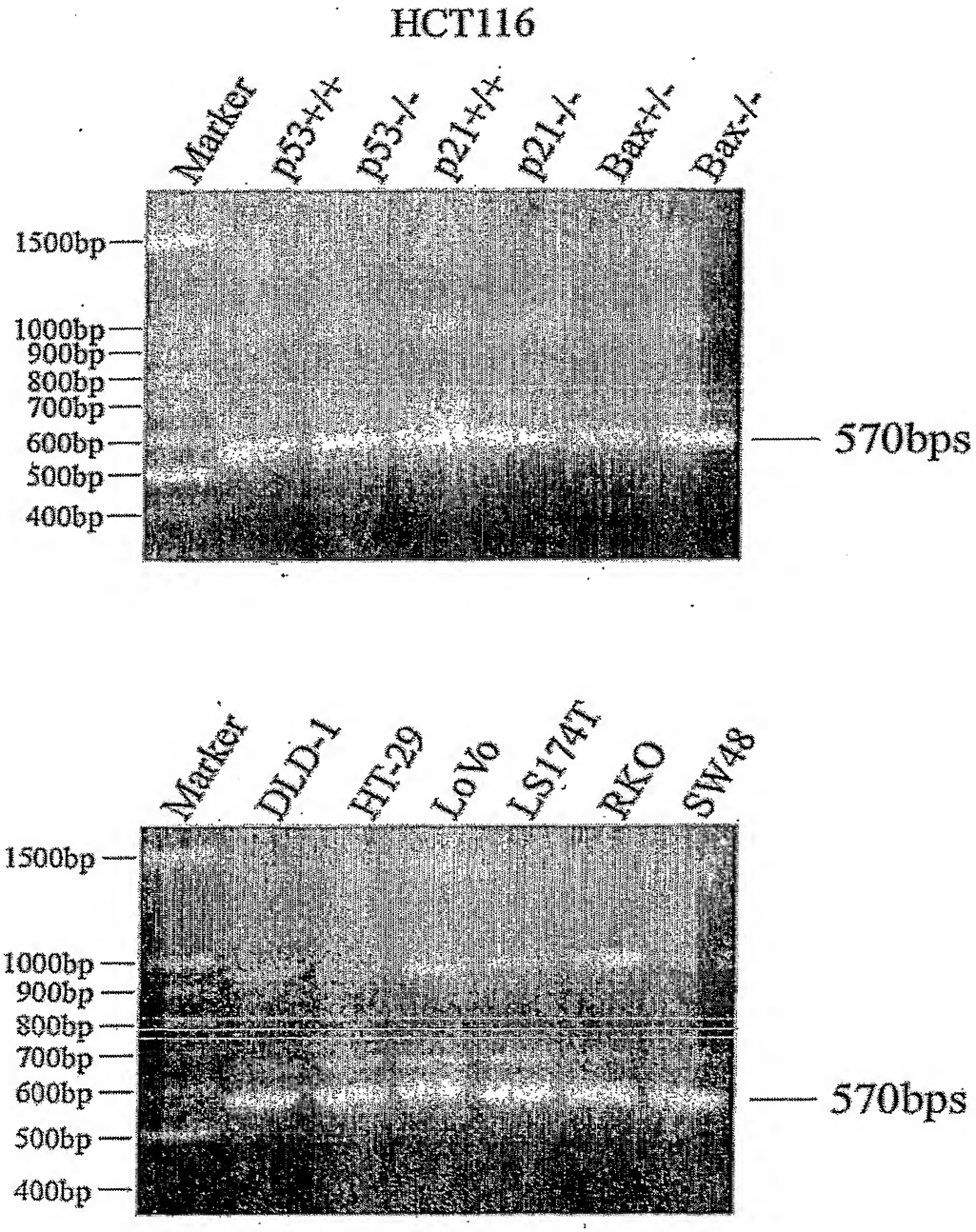

HCT116
Marker
p53+/+
p53-/-
p21+/+
p21-/-
Bax+/-
Bax-/-
1500bp
1000bp
900bp
800bp
700bp
600bp
500bp
400bp
570bps
Marker
DLD-1
HT-29
LoVo
LS174T
RKO
SW48
1500bp
1000bp
900bp
800bp
700bp
600bp
500bp
400bp
570bps

FIGURE 3

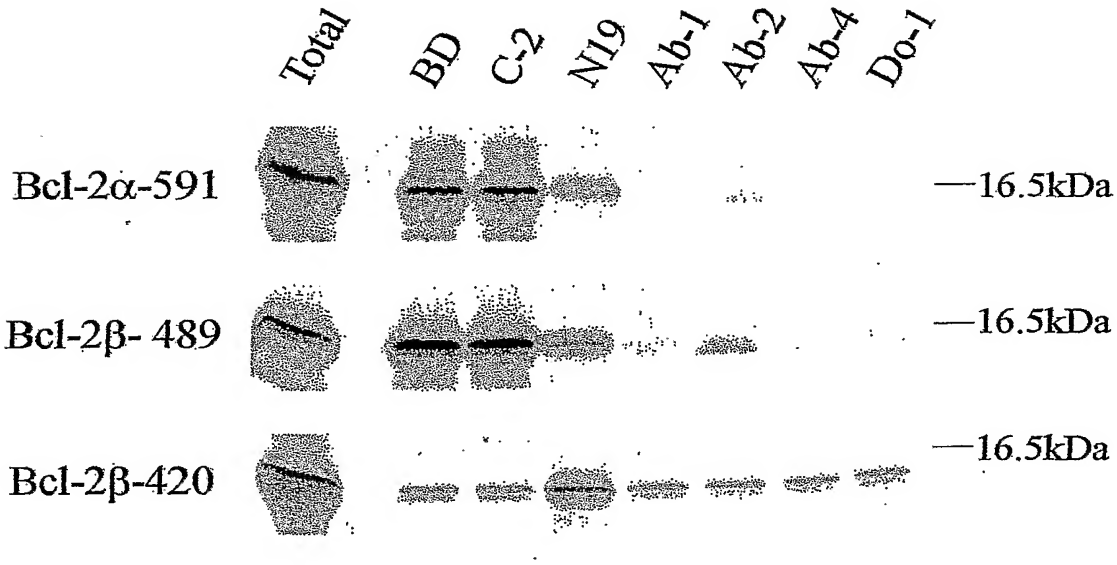
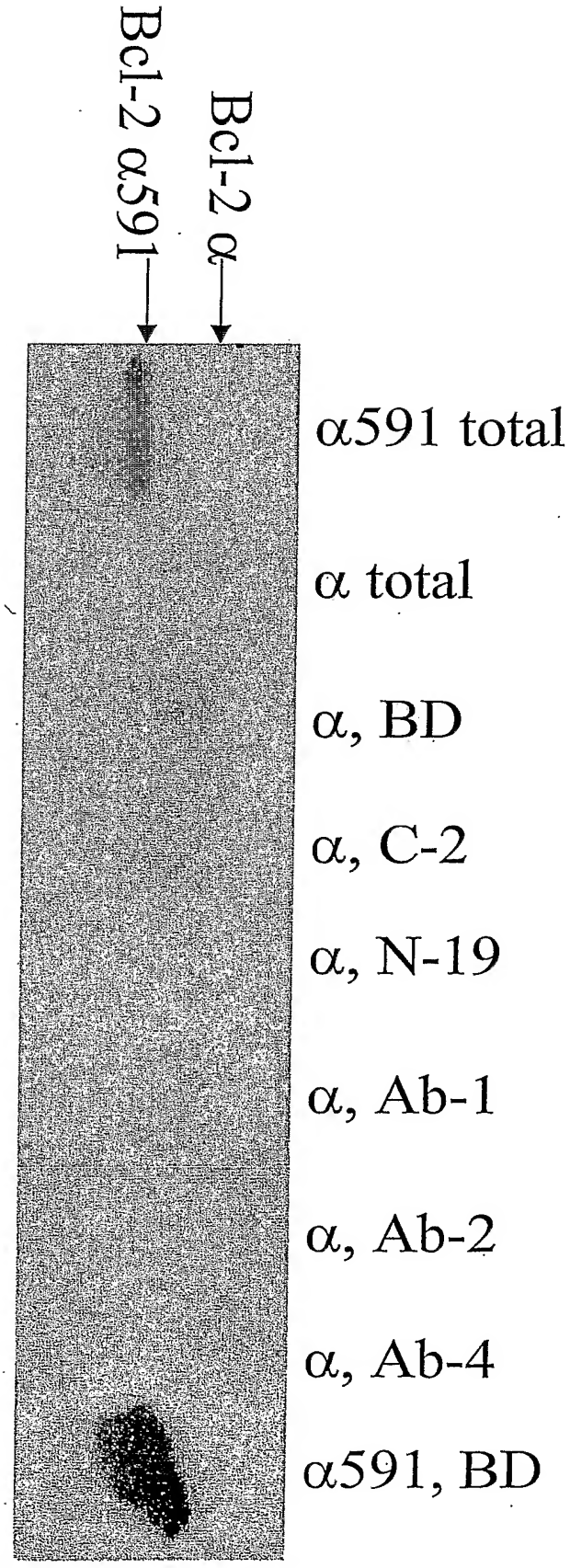

Total
BD
C-2
N19
Ab-1
Ab-2
Ab-4
Do-1
Bcl-2α-591
—16.5kDa
Bcl-2β- 489
—16.5kDa
Bcl-2β-420
—16.5kDa

Figure 4

	HCT	HCT p53-/-	HCT Bax+/-	HCT Bax-/-	HCT p21+/+	HCT P21-/-	RKO	LoVo	LS174T	SW48
α591	Φ	Φ	Φ	Φ	Φ			Φ		Φ
α588						Φ				
α480							Φ			
α633							Φ			
β489		Φ								
β474									Φ	
β420					Φ					
β315							Φ			

FIGURE 5;


Bcl-2 α591
Bcl-2 α
α591 total
α total
α, BD
α, C-2
α, N-19
α, Ab-1
α, Ab-2
α, Ab-4
α591, BD

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB2004/003326

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/82 C12N15/11 C12N15/12 C07K16/32 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CIOCA D P ET AL: "RNA interference is a functional pathway with therapeutic potential in human myeloid leukemia cell lines" CANCER GENE THERAPY, vol. 10, no. 2, February 2003 (2003-02), pages 125-133, XP002293680 ISSN: 0929-1903 the whole document	1,7-20
X	US 6 414 134 B1 (REED JOHN C) 2 July 2002 (2002-07-02) the whole document	1,2,4,5, 7,10,11, 15,17, 19,22

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 November 2004	18/11/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Andres, S

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB2004/003326

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FUTAMI T ET AL: "Induction of apoptosis in HeLa cells with siRNA expression vector targeted against bcl-2" NUCLEIC ACIDS RESEARCH SUPPLEMENT, no. 2, January 2002 (2002-01), pages 251-252, XP002968175 ISSN: 0305-1048 the whole document	1,7,8, 10-12, 17-22
X	GAUTSCHI O ET AL: "ACTIVITY OF A NOVEL BCL-2/BCL-XL-BISPECIFIC ANTISENSE OLIGONUCLEOTIDE AGAINST TUMORS OF DIVERSE HISTOLOGIC ORIGINS" JOURNAL OF THE NATIONAL CANCER INSTITUTE, vol. 93, no. 6, 21 March 2001 (2001-03-21), pages 463-471, XP009003270 ISSN: 0027-8874 the whole document	1,7,10, 11, 15-19,22
A	JIANG MING ET AL: "Bcl-2 constitutively suppresses p53-dependent apoptosis in colorectal cancer cells" GENES AND DEVELOPMENT, vol. 17, no. 7, 1 April 2003 (2003-04-01), pages 832-837, XP002293683 ISSN: 0890-9369 the whole document	1-22
A	MERCATANTE D ET AL: "Modification of alternative splicing pathways as a potential approach to chemotherapy" PHARMACOLOGY AND THERAPEUTICS, vol. 85, no. 3, March 2000 (2000-03), pages 237-243, XP002262995 ISSN: 0163-7258 the whole document	1-22

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2004/003326

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material

   [X] a sequence listing

   [ ] table(s) related to the sequence listing

   b. format of material

   [X] in written format

   [X] in computer readable form

   c. time of filing/furnishing

   [ ] contained in the international application as filed

   [ ] filed together with the international application in computer readable form

   [X] furnished subsequently to this Authority for the purpose of search

2. [X] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2004/003326

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-10 encompass methods of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB2004/003326

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6414134 B1	02-07-2002	US 6040181 A	21-03-2000
		US 5831066 A	03-11-1998
		CA 2172153 A1	30-03-1995
		EP 0722342 A1	24-07-1996
		JP 2001505401 T	24-04-2001
		JP 2003026609 A	29-01-2003
		WO 9508350 A1	30-03-1995
		US 5734033 A	31-03-1998